Pathogens and Toxins in Foods: Challenges and Interventions
Edited by V. K. Juneja and J. N. Sofos

Chapter 4

Clostridium perfringens

Vijay K. Juneja, John S. Novak, and Ronald J. Labbe

CHARACTERISTICS OF THE ORGANISM AND TYPE OF ILLNESS

Clostridium perfringens is an anaerobic (microaerophilic), gram-positive, nonmotile, spore-forming, rod-shaped bacterium. The length of the rods depends upon the growth environment. In glucose-rich medium, the rods are short, whereas in starch-based sporulation medium, the rods are long. Spores are oval and subterminal. Microbiological testing for confirmation is based upon the ability of the pathogen to hydrolyze gelatin, reduce nitrate to nitrite, and ferment lactose (stormy fermentation of lactose in milk). Lecithinase (alpha-toxin) activity also characterizes the isolates and is demonstrated by a zone of hemolysis (often a double zone) on blood agar or a pearly opalescence around colonies on egg yolk agar. This activity can be inhibited by type A antitoxin (Nagler reaction). On sulfite-containing agars, the colonies appear black due to sulfite reduction. *C. perfringens* also produces a wide variety of extracellular toxins (soluble antigens) (Table 1). The types (A, B, C, D, and E) are based upon the production of four extracellular toxins: alpha, beta, epsilon, and iota (Petit et al., 1999). These toxins are not associated with food poisoning. Types A, C, and D are pathogenic to humans. Types B, C, D, E, and maybe A affect animals. The enterotoxin produced by type A and C is distinct from the exotoxins and is responsible for the typical symptoms of food poisoning.

C. perfringens is ubiquitous in the environment and is found in soil, dust, raw ingredients, such as spices used in food processing, and in the intestines of humans and animals (ICMSF, 1996; Juneja et al., 2006a). Thus, raw protein foods of animal origin are frequently contaminated with *C. perfringens*. However, a large proportion of *C. perfringens* found in raw foods is enterotoxin negative, lacking *cpe* (Saito, 1990). In a survey conducted by Lin and Labbe (2003), none of 40 *C. perfringens* isolates from 131 retail food samples in the United States were enterotoxin-positive *C. perfringens*, containing *cpe*, whereas Wen and McClane (2004) found a prevalence rate of 1.7% in U.S. retail food. Foods that are associated with food poisoning outbreaks contain large numbers of enterotoxin-positive *C. perfringens* organisms.

The incidence of *C. perfringens* gastrointestinal illnesses in the United States has been estimated by the Centers for Disease Control and Prevention to be 248,000 cases per year, leading to 41 hospitalizations and 7 deaths each year, with 100% of these cases being due to food-borne transmission of the pathogen (Mead et al., 1999) and with an estimated cost of $200 per case (Todd, 1989). In another report from 1993 to 1997, *C. perfringens* accounted for 2.1% of the outbreaks and 3.2% of the cases of food-borne illnesses (CDC, 2000). Most cases of *C. perfringens* food poisoning are mild and are not reported. In 1994, the total cost of illnesses due to *C. perfringens* was estimated at $123 million in the United States (Anonymous, 1995). The estimated large number of illnesses due to *C. perfringens* clearly stresses the importance of cooling foods quickly after cooking, with proper refrigeration during shelf-life storage.

C. perfringens outbreaks usually result from improper handling and preparation of foods, such as inadequate cooling at the home, retail, or food service level, rarely involving commercial meat processors (Bryan, 1988; Taormina et al., 2003; CDC, 2000; Bean et al., 1997; Bean and Griffin, 1990). Major

Vijay K. Juneja • Microbial Food Safety Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19038. **John S. Novak** • American Air Liquide, Delaware Research & Technology Center, 200 GBC Drive, Newark, DE 19702-2462. **Ronald J. Labbe** • Department of Food Science, University of Massachusetts, Amherst, MA 01003-1021.

Table 1. Toxins of *Clostridium perfringens*

Lethal toxin (types)	Activity of toxins
Alpha-toxin (A, B, C, D, E)	Lethal, necrotizing, hemolytic lecithinase C
Beta-toxin (B, C)	Lethal, necrotizing
Gamma-toxin (B, C)	Lethal
Delta-toxin (B, C)	Hemolytic, lethal
Epsilon-toxin (B, D)	Lethal, necrotizing (activated by trypsin)
Eta-toxin (A)	Lethal (validity questionable)
Theta-toxin (A, B, C, D, E)	Hemolytic (oxygen labile, lethal)
Iota-toxin (E)	Necrotizing, lethal (activated by trypsin)
Kappa-toxin (A, C, D, E)	Collagenase (lethal, necrotizing, gelatinase)
Lambda-toxin (B, D, E)	Proteinase (disintegrates Azocoll and hide powder but not collagen; gelatinase)
Mu-toxin (A, B, D)	Hyaluronidase
Nu-toxin (A, B, C, D, E)	Deoxyribonuclease
Enterotoxin (A, C, D)	Complex with plasma membrane

contributing factors leading to food poisoning associated with *C. perfringens* include its ability to form heat-resistant spores that can survive commercial cooking operations, as well as the ability to germinate, outgrow, and multiply at a very rapid rate during postcook handling, primarily under conditions conducive to germination. Such conditions occur when the cooling of large batches of cooked foods is not fast enough to inhibit bacterial growth or when the foods are held at room temperature for an extended period or are temperature abused. Germination and outgrowth of *C. perfringens* spores during cooling of thermally processed meat products have been reported extensively (Juneja et al., 1994c, 1999). Accordingly, improper cooling (40.9%) of food products has been cited as the most common cause of *C. perfringens* outbreaks (Angulo et al., 1998).

C. perfringens food poisoning is one of the most common types of food-borne illness (Labbe, 1989) and occurs typically from the ingestion of $>10^6$ viable vegetative cells of the organism in temperature-abused foods (Labbe and Juneja, 2002). Acidic conditions encountered in the stomach may actually trigger the initial stages of sporulation of the vegetative cells of *C. perfringens*. Once in the small intestine, the vegetative cells sporulate, releasing an enterotoxin upon sporangial autolysis. The enterotoxin is responsible for the pathological effects in humans, as well as the typical symptoms of acute diarrhea with severe abdominal cramps and pain (Duncan and Strong, 1969; Duncan et al., 1972). Pyrexia and vomiting are usually not encountered in affected individuals. The typical incubation period before onset of symptoms is 8 to 24 hours, and acute symptoms usually last less than 24 hours. Full recovery within 24 to 48 hours is normal. Fatalities are rare in healthy individuals. Food-borne outbreaks of *C. perfringens* can be confirmed if $>10^5$ CFU/g of the organism or $>10^6$ spores/g are detected in the implicated food or feces, respectively (Labbe and Juneja, 2002).

Meat and poultry products were associated with the vast majority of *C. perfringens* outbreaks in the United States, probably due to the fastidious requirement of more than a dozen amino acids and several vitamins for the organism to grow in these products (Labbe and Juneja, 2002; Brynestad and Granum, 2002). Roast beef, turkey, meat-containing Mexican foods, and other meat dishes have been associated with *C. perfringens* food poisoning outbreaks (Bryan, 1969, 1988). Roast beef and other types of cooked beef were implicated primarily as vehicles of transmission for 26.8% of 190 *C. perfringens* enteritis outbreaks in the United States from 1973 to 1987 and 33.9% of 115 outbreaks from 1977 to 1984, although poultry products were also commonly implicated (Bean and Griffin, 1990; Bryan, 1988).

In retrospect, most outbreaks of *C. perfringens* food poisoning can be avoided by adequate cooking of meat products followed by holding at hot temperatures or rapid cooling.

SOURCES AND INCIDENCE IN THE ENVIRONMENT AND FOODS

Clostridium perfringens is commonly found on vegetable products and in other raw and processed foods. The organism is found frequently in meats, generally through fecal contamination of carcasses; contamination from other ingredients, such as spices; or postprocessing contamination. *C. perfringens* was detected in 36%, 80%, and 2% of fecal samples from cattle, poultry, and pigs, respectively (Tschirdewahn et al., 1991). The organism was found on 29, 66, and 35% of beef, pork, and lamb carcasses, respectively

(Smart et al., 1979). *C. perfringens* was isolated from 43.1% of processed and unprocessed meat samples tested in one study, including beef, veal, lamb, pork, and chicken products (Hall and Angelotti, 1965). Many areas within broiler chicken processing plants are contaminated with the organism (Craven, 2001), and the incidence of *C. perfringens* on raw poultry ranges from 10 to 80% (Waldroup, 1996). *C. perfringens* was detected in 47.4% of raw ground beef samples (Ladiges et al., 1974), and a mean level of 45.1 *C. perfringens* CFU per cm^2 was detected on raw beef carcass surface samples (Sheridan et al., 1996). *C. perfringens* was detected on 38.9% of commercial pork sausage samples (Bauer et al., 1981) and on raw beef, equipment, and cooked beef in food service establishments (Bryan and McKinley, 1979). In a survey conducted in the United Kingdom by Hobbs et al. (1965), 67% of the vacuum-packaged fish product samples were positive for clostridia, predominantly *C. perfringens*. About 50% of raw or frozen meat and poultry contains *C. perfringens* (Labbe, 1989). *C. perfringens* spores were isolated from 80% of 54 different spices and herbs (Deboer et al., 1985). This presents a public health hazard since spices and herbs are commonly used in meat and meat products. However, early surveys of foods did not determine the enterotoxin-producing ability of isolates. In recent surveys on the incidence of *C. perfringens* in raw and processed foods, an incidence level of 30 to 80% has been found (Lin and Labbe, 2003). The mild symptoms of the illness result in underreporting, as was previously explained in this chapter. Underreporting, combined with an absence of active surveillance for this microorganism, leads to only a select number of cases that are voluntarily reported to the Centers for Disease Control and Prevention in any given year. It is currently estimated that the incidence of food-borne illness from *C. perfringens* is a factor of 10 to 350 times the number of cases actually reported (Mead et al., 1999). In the United States from 1983 to 1997, *C. perfringens* was the third most common cause of confirmed outbreaks and cases of food-borne illness (CDC, 2000). There is no evidence to suggest that the contrary exists today.

Determining the level of contamination, Kalinowski et al. (2003) reported that out of 197 raw comminuted meat samples analyzed, all but 2 samples had undetectable levels (<3 spores per g) and 2 ground pork samples contained 3.3 and 66 spores per g. In another survey, Taormina et al. (2003) examined a total of 445 whole-muscle and ground or emulsified raw pork, beef, and chicken product mixtures acquired from industry sources for *C. perfringens* vegetative cells and spores. Out of 194 cured whole-muscle samples examined, 1.6% were positive for vegetative cells, and spores were not detected. Out of 152 cured ground or emulsified samples, 48.7% and 5.3% were positive for vegetative cells and spores, respectively. Populations of vegetative cells and spores did not exceed 2.72 and 2.00 $\log_{10}$ CFU/g. These studies suggest a low incidence of spores in raw, cured, whole-muscle ham, as well as low levels of spores in cured, ground, emulsified meats and in raw comminuted meat samples.

Li et al. (2007) surveyed soils and home kitchen surfaces in the Pittsburgh, PA (United States) area to determine the prevalence of *cpe*-positive *C. perfringens* isolates in these two environments and reported that neither soil nor home kitchen surfaces represent major reservoirs for type A isolates with chromosomal *cpe* that cause food poisoning, although soil does appear to be a reservoir for *cpe*-positive isolates causing non-food-borne gastrointestinal diseases. Rahmati and Labbe (2008) reported 17 samples positive for *C. perfringens,* one possessing the enterotoxin gene, out of 347 fresh and processed seafood samples examined. In another study (Wen and McClane, 2004), a survey of American retail foods did report that approximately 1.7% of raw meat, fish, and poultry items sold in retail food stores contain type A isolates carrying a chromosomal *cpe* gene, and no plasmid *cpe* gene was found in any of those surveyed retail foods. In a national survey of the retail meats conducted in Australia, *C. perfringens* was not recovered from any of the 94 ground beef samples and was isolated from 1 of 92 samples of diced lamb (Phillips et al., 2008). These surveys indicate a low incidence of *C. perfringens* in retail meats.

INTRINSIC AND EXTRINSIC FACTORS THAT AFFECT SURVIVAL AND GROWTH IN FOOD PRODUCTS AND CONTRIBUTE TO OUTBREAKS

Temperature

Although technically an anaerobe, *C. perfringens* is quite aerotolerant. The optimal growth temperature range for the organism is 43 to 45°C, although growth can occur between 15 and 50°C (Labbe and Juneja, 2002). Growth outside this range of temperatures may be characterized by extended lag and longer generation times and might require strict anaerobic conditions. Growth at 52.3°C was observed only under strict anaerobic conditions (Shoemaker and Pierson, 1976). Populations of *C. perfringens* at temperatures below 10°C have been reported to decline or remain stable, suggesting that the pathogen does not grow at proper refrigeration

temperatures (Traci and Duncan, 1974; Strong et al., 1966). Thus, refrigeration of foods contaminated with spores and vegetative cells of *C. perfringens* will not provide favorable conditions for growth.

C. perfringens is capable of extremely rapid growth in meat systems, which makes the organism a particular concern to meat processors as well as the food service industry. Hall and Angelotti (1965) found that *C. perfringens* vegetative cells inoculated into meat began multiplying without any lag phase, at 45°C. *C. perfringens* grew faster at 45°C in autoclaved ground beef than in broth media at the same temperature (Willardsen et al., 1978, 1979). One strain of *C. perfringens* had a generation time of 7.1 minutes in autoclaved ground beef held at 41°C, although the mean generation time for an eight-strain mixture ranged from 19.5 minutes at 33°C to 8.8 minutes at 45°C (Willardsen et al., 1978). Because of their rapid growth, numbers of *C. perfringens* cells sufficient to cause illness, that being $>10^6$ cells, can rapidly be reached under optimal conditions in meats and meat products. Geopfert and Kim (1975) reported that *C. perfringens* growth does not initiate in raw ground beef stored at 15°C or below, even after extended storage. However, Solberg and Elkind (1970) reported that *C. perfringens* vegetative cells in frankfurters increased by 3 $\log_{10}$ cycles in 3 days at 15°C and in 5 days at 12°C, but the growth was restricted at 10 and 5°C, respectively.

Oxygen

C. perfringens does not grow very well in the presence of oxygen. Juneja et al. (1994a, 1994b) examined growth of vegetative *C. perfringens* cells in cooked ground beef and cooked turkey at various temperatures and determined that *C. perfringens* was able to grow in both products under both anaerobic and aerobic conditions, although growth was faster in both products held under anaerobic conditions. *C. perfringens* grew in ground beef to >7 logs within 12 h at 28, 37, and 42°C under anaerobic atmosphere and at 37 and 42°C under aerobic conditions. At 28°C under aerobic conditions, growth was relatively slow and total viable count increased to >6 logs within 36 h. Similarly, growth at 15°C in air was both slower and less than that under vacuum. This suggests that aerobic conditions can delay growth of *C. perfringens,* even during temperature abuse. Regardless of packaging, the organism either declined or did not grow at 4, 8, and 12°C. Temperature abuse (28°C storage) of refrigerated products for 6 h will not permit *C. perfringens* growth (Juneja et al., 1994a). Nevertheless, temperature abuse of a product stored under aerobic conditions is unlikely to inhibit *C. perfringens* growth completely. *C. perfringens* grew in cooked turkey to about 7 logs within 9 h anaerobically and by 24 h aerobically at 28°C. While aerobic growth was slow at 15°C, mean $\log_{10}$ CFU/g increased anaerobically by 4 to 4.5 logs by day 8 for both strains (Juneja et al., 1994b).

a_w

Growth of *C. perfringens* is inhibited by water activity (a_w) ranging from 0.93 to 0.97 (Kang et al., 1969; Strong et al., 1970; Labbe and Juneja, 2006). Within this range, inhibition is dependent on the solute used to adjust the a_w and on factors such as inoculum size, pH, temperature, oxidation reduction potential, and the presence of various nutrients (Craven, 1980). While germination of spores was inhibited on laboratory medium at an a_w value of 0.93, a *C. perfringens* population at an a_w of 0.95 showed an increase of 1.8 log CFU/ml by 5 h and 4.9 $\log_{10}$ CFU/ml by 12 h at 37°C (Kang et al., 1969). Therefore, foods contaminated with low levels of *C. perfringens* would not result in substantial growth within 5 h of temperature abuse at 37°C. Growth of *C. perfringens* vegetative cells at 37°C was not observed until 12 and 20 h at a_w values of 0.965 and 0.96, respectively (Strong et al., 1970). These data suggest that low-a_w foods are unlikely to support *C. perfringens* growth sufficiently to cause illness, though growth can occur at an a_w of 0.93. Some sausages, salamis, hams, pepperonis, soups, and chipped and dried beef products have a_w values below this level (Holley et al., 1988).

pH

Optimum pH for *C. perfringens* growth is between 6.0 and 7.0, and the range is between pH 5.0 and 9.0. Some growth may be expected at pH values of ≤5.0 and ≥8.3 (Hobbs, 1979; Labbe 1989). In general, acidic foods (pH ≤5.0) would not foster growth of *C. perfringens.* However, certain *C. perfringens* strains have been found to be relatively more acid tolerant than others (de Jong, 1989). Labbe (1989) suggested that an acidic pH in foods can act synergistically with other hurdles, such as curing salts, to restrict *C. perfringens* growth.

Curing Salts

The sodium chloride level in a food affects the ability of *C. perfringens* to grow. Published research suggests that *C. perfringens* can grow in media supplemented with various levels of curing salts. While growth was not inhibited by 4% (wt/vol) NaCl, some strains do not grow in 5 to 6% NaCl, and most

strains failed to grow in 7 to 8% NaCl (Roberts and Derrick, 1978; Mead et al., 1999; Gibson and Roberts, 1986). These levels are higher than those typically found in ready-to-eat (RTE) foods.

Studies have been conducted with laboratory media on the antimicrobial effects of nitrites, with respect to the growth of *C. perfringens* vegetative cells and germination of spores (Gough and Alford, 1965; Perigo and Roberts, 1968; Labbe and Duncan, 1970; Riha and Solberg, 1975; Roberts and Derrick, 1978; Gibson and Roberts, 1986). Traditionally, sodium nitrite has been used as a preservative in cured meats to inhibit *Clostridium botulinum* growth and subsequent deadly neurotoxin production. *C. perfringens* appears to be more resistant to nitrites. Gough and Alford (1965) reported that *C. perfringens* growth was not inhibited at 8,000 ppm of sodium nitrite but was inhibited when the concentration was increased to 12,000 ppm. Further, it has been demonstrated that 0.02% and 0.01% sodium nitrite slows germination of both heat-resistant and heat-sensitive *C. perfringens* spores (Labbe and Duncan, 1970; Sauter et al., 1977).

It is worth mentioning that the inhibitory effect of sodium nitrite is enhanced at elevated temperatures (Davidson and Juneja, 1990), since heat-injured spores are more sensitive to the effects of nitrite (Chumney and Adams, 1980; Labbe and Duncan, 1970). Anaerobic conditions can also increase the antimicrobial effectiveness of sodium nitrite. Further, the inhibitory effect of nitrite can be enhanced under acidic conditions and by prior heating of the medium containing nitrite (Labbe, 1989; Riha and Solberg, 1975).

The ability of *C. perfringens* to grow in many cured meats is well documented. For instance, *C. perfringens* spores grew rapidly in frankfurters at 37°C and 23°C, and although growth was slower at lower temperatures, populations increased 2 log CFU/g after 2 days at 15°C and 3 days at 12°C (Solberg and Elkind, 1970). Most processed meat products contain about 2.75 and 3.25% salt (Maurer, 1983), and sodium nitrite is allowed in cured meats at ingoing (initial) levels of 156 ppm. Vareltzis et al. (1984) found that chicken frankfurters containing 2.6% salt and 150 ppm sodium nitrite did not allow *C. perfringens* growth from spores (starting inoculum of 4.7 log) over 9 days at 20°C. Hallerbach and Potter (1981) reported that frankfurters containing 2.2% salt and 140 ppm nitrite did not support *C. perfringens* vegetative cell growth for 74 h at 20°C. Further, Amezquita et al. (2004) reported no growth of *C. perfringens* at 21 and 17°C after 10 and 21 days, respectively, in cured ham (initial sodium nitrite concentration of 156 ppm). Gibson and Roberts (1986) reported that the inhibitory concentrations of sodium nitrite can be lowered if combined with other curing salts. In their study, *C. perfringens* growth at 20°C was inhibited by 200 μg of nitrite/ml and 3% NaCl or 50 μg of nitrite/ml and 4% NaCl at pH 6.2 in a laboratory medium. In another study, the levels of sodium nitrite necessary to inhibit the strains tested dropped from 300 ppm to 25 ppm when the concentration of NaCl was increased from 3 to 6% (Roberts and Derrick, 1978). Thus, sodium nitrite acts synergistically with NaCl to inhibit *C. perfringens* growth. The differences in the minimum growth temperatures could be a function of product characteristics, such as meat species, pH, and concentrations of functional ingredients, such as NaCl, phosphates, and other antimicrobial ingredients, such as nitrates/nitrites or salts of organic acids. These ingredients have been shown to affect the growth of *C. perfringens* from spore inocula from specific meat products (Juneja and Thippareddi, 2004a, 2004b; Thippareddi et al., 2003; Zaika 2003).

FOOD PROCESSING OPERATIONS THAT INFLUENCE THE NUMBERS, SPREAD, OR CHARACTERISTICS

Slow cooking associated with low-temperature, long-time cooking will not eliminate *C. perfringens* spores. Cooking temperatures, if designed to inactivate *C. perfringens* spores, may negatively impact the product quality and desirable organoleptic attributes of foods. Therefore, spores are likely to survive the normal pasteurization/cooking temperatures applied to these foods. Cooking usually increases the anaerobic environment in food and reduces the numbers of competing spoilage organisms, which is ecologically important because *C. perfringens* competes poorly with the spoilage flora of many foods. Mean generation times in autoclaved ground beef during slow heating from 35 to 52°C ranged from 13 to 30 minutes, with temperature increases of 6 to 12.5°C/hour (Willardsen et al., 1978). Another study also demonstrated growth of the organism in autoclaved ground beef during linear temperature increases (4.1°C to 7.5°C/hour) from 25°C to 50°C. (Roy et al., 1981).

Cooking of foods can also heat shock *C. perfringens* spores, since germination activation of *C. perfringens* spores can occur at temperatures between 60 and 80°C (Walker, 1975). Similar to spores of other bacterial species, spores of *C. perfringens* germinate at a higher rate after heat shock. For instance, while only 3% of inoculated *C. perfringens* spores germinated in raw beef without prior heat shock, almost all spores germinated after the beef received a heat treatment

(Barnes et al., 1963). As a result of such heat shock conditions, a physiological response is triggered in organisms, leading to the synthesis of a specific set of proteins known as heat shock proteins (HSPs). Synthesis of HSPs has been observed in bacterial as well as mammalian cells (Lindquist and Craig, 1988). After heat shock, germination and outgrowth of spores and *C. perfringens* vegetative growth are likely to occur in cooked foods if the rate and extent of cooling are not sufficient or if the processed foods are temperature abused. The abuse may occur during transportation, distribution, storage, or handling in supermarkets, or during preparation of foods by consumers which includes low-temperature–long-time cooking of foods as well as scenarios in which foods are kept on warming trays before final heating or reheating. Studies have described growth of *C. perfringens* during cooling of cooked, uncured meat products. In a study by Tuomi et al. (1974), when cooked ground beef gravy inoculated with a mixture of vegetative cells and spores of *C. perfringens* NCTC 8239 was cooled in a refrigerator, rapid growth of the organism was reported to occur during the first 6 h of cooling, when the gravy temperature was in an ideal growth temperature range. While the study by Tuomi et al. (1974) identified the cooling stage as most critical in ensuring the safety of such products, the experimental design included both the vegetative cells and spores of *C. perfringens;* the vegetative cells would not likely be present in a cooked product. Shigehisa et al. (1985) reported on germination and growth characteristics of *C. perfringens* spores inoculated into ground beef at 60°C and cooled to 15°C at a linear cooling rate of 5 to 25°C/h. They observed that the organism did not grow during exposure to falling temperature rates of 25 to 15°C/h. However, multiplication of the organism was observed when the rate was less than 15°C/h. Interestingly, the total population did not change for the first 150 min, regardless of the cooling rate. This study is not totally applicable to typical retail food operations because cooling is not linear; it is exponential.

Steele and Wright (2001) evaluated growth of *C. perfringens* spores in turkey roasts cooked to an internal temperature of 72°C and then cooled in a walk-in cooler from 48.9°C to 12.8°C in 6, 8, or 10 h. Results of that study indicated that an 8.9-hour cooling period was adequate to prevent growth of *C. perfringens* with a 95% tolerance interval. To simulate commercial chili cooling procedures, Blankenship et al. (1988) conducted exponential cooling experiments in which the cooling time was 4 and 6 h for a temperature decline from 50 to 25°C. This is approximately equivalent to a cooling rate of 12 h and 18 h for temperatures of 54.4 to 7.2°C. They observed a declining growth rate in the case of 4-h and 6-h cooling times. Juneja et al. (1994c) reported that no appreciable growth (<1.0 $\log_{10}$ CFU/g) occurred if cooling took 15 h or less when cooked ground beef inoculated with heat-activated *C. perfringens* spores was cooled from 54.4 to 7.2°C at an exponential rate, that being more rapid cooling at the beginning followed by a slower cooling rate later. However, *C. perfringens* grew by 4 to 5 $\log_{10}$ CFU/g if the cooling time was greater than 18 h. This implies that *C. perfringens* is capable of rapid growth in meat systems, making this organism a particular concern to meat processors, as well as to the food service industry.

A limited amount of published research is available regarding growth of the pathogen in cooked cured meats during cooling. Taormina et al. (2003) inoculated bologna and ham batter with *C. perfringens* spores and then subjected them to cooking and either cooling procedures typically used in industry or extended chilling. In that study, growth of the organism was not detected in any of the products tested during chilling from 54.4 to 7.2°C. Zaika (2003) reported complete inhibition of *C. perfringens* germination and growth in cured hams with NaCl concentrations of 3.1%, when cooled exponentially from 54.4 to 7.2°C within 15, 18, or 21 h. Cooked cured turkey cooled from 48.9 to 12.8°C did not support *C. perfringens* growth in 6 h; however, a 3.07 log increase was observed following a 24-h cooling time (Kalinowski et al., 2003).

RECENT ADVANCES IN BIOLOGICAL, CHEMICAL, AND PHYSICAL INTERVENTIONS TO GUARD AGAINST THE PATHOGEN

The presence of inhibitory agents in the products can affect germination of *C. perfringens* spores and may also affect the minimum growth temperatures for the germinated spores. Recent studies have shown the efficacy of certain antimicrobial agents against the growth of *C. perfringens* during cooling of meat products. For instance, Sabah et al. (2003) found that 0.5 to 4.8% sodium citrate inhibited growth of *C. perfringens* in cooked, vacuum-packaged, restructured beef cooled from 54.4°C to 7.2°C within 18 h. The same researchers also demonstrated growth inhibition of the microorganism by oregano in combination with organic acids during cooling of sous-vide-cooked ground beef products (Sabah et al., 2004). Organic acid salts, such as 1 to 1.5% sodium lactate, 1% sodium acetate, 0.75 to 1.3% buffered sodium citrate (with or without sodium diacetate), and 1.5% sodium lactate supplemented with sodium diacetate,

inhibited germination and outgrowth of *C. perfringens* spores during the chilling of marinated ground turkey breast (Juneja and Thippareddi, 2004a, 2004b). In another study, Thippareddi et al. (2003) reported complete inhibition of *C. perfringens* spore germination and outgrowth by sodium salts of lactic and citric acids (2.5 and 1.3%, respectively) in roast beef, pork ham, and injected turkey products. Incorporation of plant-derived natural antimicrobials, such as thymol (1 to 2%), cinnamaldehyde (0.5 to 2%), oregano oil (2%), and carvacrol (1 to 2%), as well as the biopolymer chitosan (0.5 to 3%) derived from shellfish and green tea catechins (0.5 to 3%), individually inhibited *C. perfringens* germination and outgrowth during exponential cooling of ground beef and turkey (Juneja et al., 2006a, 2006b, 2007; Juneja and Friedman, 2007). Therefore, natural compounds can be used as ingredients in processed meat products to provide an additional measure of safety to address the *C. perfringens* hazard during chilling and subsequent refrigeration of meat products, thus further minimizing risk to the consumer.

Numerous studies have examined the heat resistance of *C. perfringens* spores and/or vegetative cells. Heat resistance varies among strains of *C. perfringens*, although both heat-resistant and heat-sensitive strains can cause food poisoning (Labbe and Juneja, 2006). Environmental stresses, such as storage and holding temperatures and low-temperature–long-time cooking, expose the contaminating vegetative and spore-forming food-borne pathogens to conditions similar to heat shock, thereby rendering the heat-shocked organisms more resistant to subsequent lethal heat treatments. Researchers have reported on the quantitative assessment of heat resistance, the heat shock response, and the induced thermotolerance to assist food processors in designing thermal processes for the inactivation of *C. perfringens*, thereby ensuring the microbiological safety of cooked foods (Juneja et al., 2001a, 2003). Heat shocking vegetative cells suspended in beef gravy at 48°C for 10 min allowed the microorganisms to survive longer and increased the heat resistance by as high as 1.5-fold (Juneja et al., 2001a). The thermal resistance (*D* values in min) of *C. perfringens* cells heated in beef gravy at 58°C ranged from 1.21 min (C 1841 isolate) to 1.60 min (F 4969 isolate). Compared to the control (no heat shock), the increase in heat resistance after heat shocking at 58°C ranged from 1.2-fold (B 40 isolate) to 1.5-fold (NCTC 8239 isolate). The *D* values of *C. perfringens* spores heated in beef gravy at 100°C ranged from 15.50 min (NCTC 8239 isolate) to 21.40 min (NB 16 isolate) (Juneja et al., 2003). Compared to the control (no heat shock), the increase in heat resistance of *C. perfringens* spores at 100°C after heat shocking ranged from 1.1-fold (NCTC 8238 isolate) to 1.5-fold (F 4969 isolate). Similar results were obtained by Heredia et al. (1997), who found that a sublethal heat shock at 55°C for 30 minutes increased tolerance of both spores and vegetative cells to a subsequent heat treatment. In their study, when the heat resistance of *C. perfringens* vegetative cells, grown at 43°C in fluid thioglycolate medium to an A_{600} of 0.4 to 0.6, was determined, *D* values at 55°C of 9 and 5 min for the FD-1 and FD1041 strains, respectively, were reported. The *D* values of the heat-shocked cells were 85 and 10 min, respectively. Heredia et al. (1997) heat shocked sporulating cells of *C. perfringens* at 50°C for 30 min and then determined the *D* values at 85 or 90°C. The authors reported that a sublethal heat shock increased the thermotolerance of *C. perfringens* spores by at least 1.7- to 1.9-fold; the *D* values at 85°C increased from 24 to 46 min and at 95°C from 46 to 92 min, respectively. Bradshaw et al. (1982) reported that *D* values at 99°C for *C. perfringens* spores suspended in commercial beef gravy ranged from 26 to 31.4 min. Miwa et al. (2002) found that spores of enterotoxin-positive *C. perfringens* strains were more heat resistant than enterotoxin-negative strains. Similarly, food poisoning isolates of the organism are generally more heat resistant than *C. perfringens* spores from other sources (Labbe, 1989). Sarker et al. (2000) reported *D* values at 100°C for 12 isolates of *C. perfringens* spores, carrying either the chromosomal *cpe* gene or the plasmid *cpe* gene, in DS medium ranged from 0.5 min to 124 min. Sarker et al. (2000) reported *D* values at 55°C for *C. perfringens* cells grown at 37°C in FTG of 12.1 min and 5.6 min for the E13 and F5603 strains, respectively. These authors also reported a strong association of the food poisoning isolates and increased heat resistance; the *D* values at 55 or 57°C for the *C. perfringens* chromosomal *cpe* isolates were significantly higher ($P < 0.05$) than the *D* values of the *C. perfringens* isolates carrying a plasmid *cpe* gene; however, differences in heat resistance levels were not observed at higher temperatures. Nevertheless, understanding these variations in heat resistance is certainly necessary in order to design adequate cooking regimes to eliminate *C. perfringens* vegetative cells in RTE foods.

Studies have been conducted to assess the effects and interactions of multiple food formulation factors on the heat resistance of spores and vegetative cells of *C. perfringens*. In a study by Juneja and Marmer (1996), when the thermal resistance of *C. perfringens* spores (expressed as *D* values in min) in turkey slurries that included 0.3% sodium pyrophosphate at pH 6.0 and salt levels of 0, 1, 2, or 3% was assessed, the *D* values at 99°C decreased from 23.2 min (no salt)

to 17.7 min (3% salt). In a beef slurry, the *D* values significantly decreased ($P < 0.05$) from 23.3 min (pH 7.0, 3% salt) to 14.0 min (pH 5.5, 3% salt) at 99°C (Juneja and Majka 1995). While addition of increasing levels (1 to 3%) of salt in turkey (Juneja and Marmer, 1996) or a combination of 3% salt and pH 5.5 in beef (Juneja and Majka, 1995) can result in a parallel increase in the sensitivity of *C. perfringens* spores at 99°C, mild heat treatments given to minimally processed foods will not eliminate *C. perfringens* spores. Juneja and Marmer (1998) examined the heat resistance of vegetative *C. perfringens* cells in ground beef and turkey containing sodium pyrophosphate (SPP). The *D* values for beef that included no SPP were 21.6, 10.2, 5.3, and 1.6 min at 55, 57.5, 60, and 62.5°C, respectively; the values for turkey ranged from 17.5 min at 55°C to 1.3 min at 62.5°C. Addition of 0.15% SPP resulted in a concomitant decrease in heat resistance, as evidenced by reduced bacterial *D* values. The *D* values for beef that included 0.15% SPP were 17.9, 9.4, 3.5, and 1.2 min at 55, 57.5, 60, and 62.5°C, respectively; the values for turkey ranged from 16.2 min at 55°C to 1.1 at 62.5°C. The heat resistance was further decreased when the SPP levels in beef and turkey were increased to 0.3%. Heating such products to an internal temperature of 65°C for 1 min killed >8 $\log_{10}$ CFU/g. The *z* values for beef and turkey for all treatments were similar, ranging from 6.22 to 6.77°C. Thermal death time values from this study should assist institutional food service settings in designing thermal processes that ensure safety against *C. perfringens* in cooked beef and turkey.

Researchers have assessed the efficacy of added preservatives on inhibiting or delaying the growth of *C. perfringens* in extended shelf-life, refrigerated, processed meat products. When ground turkey containing 0.3% SPP and 0, 1, 2, or 3% salt was sous vide processed (71.1°C) and held at 28°C, lag times of 7.3, 10.6, 11.6, and 8.0 h were observed for salt levels of 0, 1, 2, and 3%, respectively (Juneja and Marmer 1996). Growth of *C. perfringens* spores in cooked ground turkey with added 0.3% SPP was inhibited for 12 h at 3% salt, pH 6.0, and 28°C. After 16 h, spores germinated and grew at 28°C from 2.25 to >5 $\log_{10}$ CFU/g in sous-vide-processed (71.1°C) turkey samples, regardless of the presence or absence of salt (Juneja and Marmer, 1996). While *C. perfringens* spores germinated and grew at 15°C to >5 $\log_{10}$ CFU/g in turkey with no salt by day 4, the presence of 3% salt in samples at 15°C completely inhibited germination and subsequent multiplication of vegetative cells, even after 7 days of storage (Juneja and Marmer, 1996). Thus, the addition of 3% salt in sous-vide-processed ground turkey containing 0.3% SPP delayed growth for 12 h at 28°C and completely inhibited the outgrowth of spores at 15°C (Juneja and Marmer, 1996). However, 3% salt in RTE products will not inhibit germination and growth of *C. perfringens* spores if refrigerated products are temperature abused to 28°C for an extended period. In another study (Juneja and Majka, 1995), the combination of 3% salt and pH 5.5 inhibited *C. perfringens* growth from spores in sous-vide-processed ground beef supplemented with 0.3% SPP at 15 and 28°C. Growth from *C. perfringens* spores occurred within 6 days in sous-vide-processed (71.1°C and pH 7.0) ground beef samples but was delayed until day 8 in the presence of 3% salt at pH 5.5 at 15°C (Juneja and Majka, 1995). *C. perfringens* growth from a spore inoculum at 4°C was not observed with sous-vide-cooked turkey or beef samples (Juneja and Marmer, 1996; Juneja and Majka, 1995). In a related study, Juneja et al. (1996) showed that *C. perfringens* growth in cooked turkey can be effectively inhibited in an atmosphere containing 25 to 75% CO_2, 20% O_2, and a balance of N_2 in conjunction with good refrigeration; however, the atmosphere cannot be relied upon to eliminate the risk of *C. perfringens* food poisoning in the absence of proper refrigeration (Juneja et al., 1996). Kalinowski et al. (2003) investigated the fate of *C. perfringens* in cooked-cured and uncured turkey at refrigeration temperatures. In their study, *C. perfringens* decreased by 2.52, 2.54, and 2.75 $\log_{10}$ CFU/g in cured turkey held at 0.6, 4.4, and 10°C, respectively, and the reductions in levels were similar in uncured turkey.

The efficacy of sodium lactate (NaL) in inhibiting the growth from spores of *C. perfringens* in a sous-vide-processed food has been assessed. Inclusion of 3% NaL in sous vide beef goulash inhibited *C. perfringens* growth at 15°C, delayed growth for a week at 20°C, and had little inhibitory effect at 25°C (Aran, 2001). While addition of 4.8% NaL restricted *C. perfringens* growth from spores for 480 h at 25°C in sous-vide-processed (71.1°C) marinated chicken breast, it delayed growth for 648 h at 19°C. *C. perfringens* growth was not observed at 4°C, regardless of NaL concentration (Juneja, 2006). These studies suggest that NaL can have significant bacteriostatic activity against *C. perfringens* and may provide sous-vide-processed foods with a degree of protection against this microorganism, particularly if employed in conjunction with adequate refrigeration.

Predictive bacterial growth models that describe *C. perfringens* spore germination and outgrowth during cooling of food systems have been generated by researchers using constant temperature data. Juneja et al. (1999) presented a model for predicting the relative growth of *C. perfringens* from spores, through lag, exponential, and stationary phases of growth, at

temperatures spanning the entire growth temperature range of about 10 to 50°C. Huang (2003a, 2003b, 2003c) used different mathematical methods to estimate the growth kinetics of *C. perfringens* in ground beef during isothermal, square-waved, linear, exponential, and fluctuating cooling temperature profiles. Juneja et al. (2001b) developed a predictive cooling model for cooked, cured beef based on growth rates of the organism at different temperatures, which estimated that exponential cooling from 51 to 11°C in 6, 8, or 10 h would result in an increase of 1.43, 3.17, and 11.8 $\log_{10}$ CFU/g, respectively, when assuming that the ratio of the mathematical lag time to the generation time for cells in the exponential phase of growth was equal to 8.068, the estimated geometric mean. A similar model was later developed for cooked, cured chicken (Juneja and Marks, 2002). Juneja et al. (2006c) developed a model for predicting growth of *C. perfringens* from spore inocula in cured pork ham. In their study, isothermal growth of *C. perfringens* at various temperatures from 10 to 48.9°C was evaluated using a methodology that employed a numerical technique to solve a set of differential equations, simulating the dynamics of bacterial growth; the authors described the effect of temperature on the kinetic parameter *K* (dissociation constant) by the modified Ratkowski model. According to the coefficient of the model, the estimated theoretical minimum and maximum growth temperatures of *C. perfringens* in cooked cured pork were 13.5 and 50.6°C, respectively. The kinetic and growth parameters obtained from these studies can be used in evaluating growth of *C. perfringens* from spore populations during dynamically changing temperature conditions, such as those encountered in meat processing.

In a model for growth of *C. perfringens* during cooling of cooked uncured beef (Juneja et al., 2008), for a temperature decline from 54.4°C to 27°C in 1.5 h, the models predicted a $\log_{10}$ relative growth of about 1.1, with a standard error of about 0.08 $\log_{10}$, while observed results for two replicates were 0.43 and 0.90 $\log_{10}$; for the same temperature decline in 3 h, the predicted $\log_{10}$ relative growth was about 3.6 $\log_{10}$ (with a standard error of about 0.07), and the observed $\log_{10}$ relative growths were 2.4 and 2.5 $\log_{10}$. When the cooling scenarios extended to lower temperatures, the predictions were somewhat better, taking into account the larger relative growth. For a cooling scenario of 54.4°C to 27°C in 1.5 h and 27°C to 4°C in 12.5 h, the average observed and predicted $\log_{10}$ relative growths were 2.7 $\log_{10}$ and 3.2 $\log_{10}$, respectively; when cooling was extended from 27°C to 4°C in 15 h, the average observed and predicted $\log_{10}$ relative growths were 3.6 $\log_{10}$ and 3.7 $\log_{10}$, respectively. For the latter cooling scenario the levels were greater than 6 $\log_{10}$, still less than stationary levels of about 7 or 8 $\log_{10}$. The differences of the estimates obtained for the models were insignificant. Smith-Simpson and Schaffner (2005) collected data under changing temperature conditions and developed a model to predict growth of *C. perfringens* in cooked beef during cooling. It was suggested that the accuracy of the germination, outgrowth, and lag time model has a profound influence upon the overall prediction, with small differences in the germination, outgrowth, and lag time prediction (~1 h) having a very large effect on the predicted final concentration of *C. perfringens*. Amezquita et al. (2004) developed an integrated model for heat transfer and dynamic growth of *C. perfringens* during cooling of cured ham and demonstrated that the effective integration of engineering and microbial modeling is a useful quantitative tool for ensuring microbiological safety. The above models can be successfully used to design microbiologically "safe" cooling regimes for cooked meat and poultry products.

Recent research has focused on combining traditional inactivation, survival, and growth-limiting factors at subinhibitory levels with emerging novel nonthermal intervention food preservation techniques using ionizing radiation, high hydrostatic pressure, or exposure to ozone. For example, the efficacy of high pressure is considerably enhanced when combined with heat, antimicrobials, or ionizing radiation. The effect of the combined intervention strategies is either additive or synergistic, in which the interaction leads to a combined effect of greater magnitude than the sum of the constraints applied individually. For example, the lethal effect of heat on spores can be enhanced after exposure to ozone. Novak and Yuan (2004) reported that the spores were more sensitive to heat at 55 or 75°C following 5 ppm of aqueous ozone for 5 min. Shelf-life extension of meat processed with 5 ppm O_3 for 5 min and containing *C. perfringens* spores combined with modified atmosphere packaging as a "hurdle" technology was proven to be effective in inhibiting spore germination and outgrowth over 10 days of storage at CO_2 concentrations above 30% and 4°C (Novak and Yuan, 2004). Likewise, *C. perfringens* cells exposed to 3 ppm O_3 for 5 min following mild heat exposure (55°C for 30 min) were more susceptible to ozone treatment.

When *C. perfringens* spores were suspended in peptone solution and exposed to combination treatments of hydrostatic pressure (138 to 483 MPa), time (5 min), and temperature (25 to 50°C), inactivation of spores ranged between 0.1 and 0.2 log cycles (Kalchayanand et al., 2004). When suspended spores

were pressurized at 50°C for 5 min and stored at 4°C or 25°C for 24 h, 12 to 52% spores germinated, indicating that germination increased both at 4°C and 25°C during 24 h. Log_{10} reductions of spores were 6.1 log/ml when bacteriocins were supplemented in the recovery medium. These results show that germinated spores at high levels could be killed by using a bacteriocin-based preservative in foods.

Regulatory Requirements

Due to its ubiquitous nature and rapid growth in meat products, *C. perfringens* can be a potential hazard in processed meat and poultry products. The FDA (2001) Food Code dictates that cooked potentially hazardous foods, such as meats, should be cooled from 60 to 21°C within 2 h, and from 60 to 5°C within 6 h. In the United Kingdom, it is recommended that uncured cooked meats be cooled from 50 to 12°C within 6 h and from 12 to 5°C within 1 h (Gaze et al., 1998). Safe cooling times for cured meats may be up to 25% longer (Gaze et al., 1998). The USDA/FSIS compliance guidelines (USDA/FSIS, 2001) for chilling of thermally processed meat and poultry products state that these products should be chilled according to the prescribed chilling rates or require that process authorities validate the safety of customized chilling rates to control spore-forming bacteria. Specifically, the guidelines state that cooling from 54.4 to 26.7°C should take no longer than 1.5 h and that cooling from 26.7 to 4.4°C should take no longer than 5 h (USDA/FSIS, 2001). Additional guidelines allow for the cooling of certain cured cooked meats from 54.4 to 26.7°C in 5 h and from 26.7 to 7.2°C in 10 h (USDA/FSIS, 2001). If meat processors are unable to meet the prescribed time-temperature cooling schedule, they must be able to document that the customized or alternative cooling regimen used will result in a less than 1-log_{10}-CFU increase in *C. perfringens* in the finished product. If the cooling guidelines cannot be achieved, computer modeling and/or product sampling can be used to evaluate the severity and microbiological risk of the process deviation, and additional challenge studies may be necessary to determine whether performance standards have been met.

Detection of *C. perfringens* in Foods

The ability of *C. perfringens* to cause food-borne illness and occasional associated outbreaks necessitates effective discriminatory detection methods for this pathogen in order to ensure reliable and confirmatory epidemiological screening of suspected foods. The many available methods of detection can be categorized as (i) metabolite-based biochemical (phenotyping/biotyping) assays, (ii) toxin, antigen-based immunological methods, and (iii) nucleic acid-based molecular techniques. Each characterization scheme also contains various advantages and disadvantages, with respect to sensitivity and discrimination against similar related clostridia, ease of analysis, time to results, and expense of reagents and/or equipment and materials (Table 2).

According to the U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition (FDA/CFSAN), *Bacteriological Analytical Manual*, methods are prescribed for the enumeration and identification of *C. perfringens* in foods (Rhodehamel and Harmon, 1998). These methods are based upon the microorganism's innate ability to reduce sulfite to sulfide, producing a distinctive black ferrous sulfide precipitate on tryptose-sulfite-cycloserine (TSC) agar (Harmon et al., 1971). With added egg yolk emulsion, *C. perfringens* colonies produce a 2- to 4-mm opaque white zone surrounding the colonies, as a result of lecithinase activity (Rhodehamel and Harmon, 1998). Further presumptive tests include rapid coagulation of an iron-milk medium, liquefaction of gelatin in lactose-gelatin medium, fermentation of lactose-producing acid and gas, lack of motility in motility-nitrate medium, and an ability to reduce nitrates to nitrites (Rhodehamel and Harmon, 1998).

Oxoid Ltd. (Basingstoke, Hampshire, United Kingdom) has recently developed a chromogenic medium for identification and enumeration of *C. perfringens* colonies in water samples and claims to provide increased sensitivity and specificity compared to TSC agar (Oxoid Ltd., 2002). The chromogenic m-CP medium differentiates yellow colonies of *C. perfringens* based upon the microorganism's ability to ferment sucrose, whereas non-*C. perfringens* colonies that hydrolyze indoxyl-β-D-glucoside turn purple. Additionally, exposure to ammonium hydroxide causes a dark pink color change in phosphatase-producing colonies (Oxoid Ltd., 2002). A recent European patent, EP1816209, describes a new medium for selective differentiation of *C. perfringens* that utilizes sodium bisulfite and ammonium ferric citrate to distinguish sulfite reduction capability combined with a fluorogenic substrate, 4-methylumbelliferyl phosphate disodium salt, which is used for acid phosphatase detection (Araujo, 2007).

Different media are continually being compared for their use in the enumeration of *C. perfringens* bacteria from foods. A recent report found that TSC was still the medium of choice when compared with iron sulfite agar, Shahidi-Ferguson perfringens agar, sulfite cycloserine azide (SCA), differential clostridial agar (DCA), and oleandomycin polymyxin sulfadiazine perfringens agar (de Jong et al., 2003). Although all

Table 2. Comparison of commonly used discriminatory methods for detection of *C. perfringens*[a]

Method	Detection limits	Considerations	Reference
Mouse neutralization	6 MLD_{50}/ml	Inhumane and costly	Uzal et al. (2003)
ELISA	5 ng CPE/g feces	Sporulation variability in foods	Bartholomew et al. (1985)
4-Layer ELISA	6.25 ng/ml toxin	Proteolysis	El Idrissi (1989)
PC-ELISA	0.075 MLD_{50}/ml	False positives	Uzal et al. (2003)
MC-ELISA	0.75 MLD_{50}/ml	Lower sensitivity	Uzal et al. (2003)
CIEP	200 MLD_{50}/ml	False negatives	Uzal et al. (2003)
Vero cell assay	40 ng CPE/g feces	Sensitivity and reproducibility	Berry et al. (1988)
Reversed passive latex agglutination	50–100 ng CPE/g feces	Nonspecific interference	Brett (1998)
Colony hybridization	10 CFU/g food	48-h detection	Baez and Juneja (1995)
Hydrophobic grid membrane filter	10 CFU/g food	Isolation of *cpe*-positives	Heikinheimo et al. (2004)
Real-time PCR	50 fg genomic DNA 20 cells (pure culture)	Unidentified PCR inhibitors	Wise and Siragusa (2005)
Multiplex PCR	Enrichment culture	False positives	Baums et al. (2004)
PFGE	DNA preparations from enriched samples	Problematic nucleases	Maslanka et al. (1999)
AFLP	DNA preparations from enriched samples	Multiple genetic elements	Keto-Timonen et al. (2006)
Ribotyping	DNA preparations from enriched samples	Costly equipment and existing databases	Schalch et al. (1997)
Oligonucleotide microarrays	DNA preparations from enriched samples	Fluorescently labeled DNA probes hybridized to single-stranded DNA on a chip	Al-Khaldi et al. (2004)

[a]This list is representative of the many types of methods available, and although it may not be totally inclusive, many other methods that are not listed here may represent only small variations or improvements over the techniques represented here.

of the media tested were effective for enumeration studies, SCA and DCA were less effective at low concentrations of *C. perfringens* in food (de Jong et al., 2003). Shahidi Ferguson perfringens and oleandomycin polymyxin sulfadiazine perfringens agars produced low counts at times, and DCA and SCA were very laborious to prepare (de Jong et al., 2003). Iron sulfite agar lacked selectivity (de Jong et al., 2003).

In another study, fluorocult-supplemented TSC agar was compared with TSC agar, m-CP agar, tryptose-sulfite-neomycin agar, sulfite polymyxin sulfadiazine agar, and Wilson-Blair agar for detection and enumeration of *C. perfringens* spores in water (Araujo et al., 2004). It was determined that membrane filtration with fluorogenic TSC agar performed better than any of the other tested enumeration media for *C. perfringens* (Araujo et al., 2004). In addition, a low confirmation rate for *C. perfringens* on m-CP medium was explained by the very subjective color differentiation of pink colonies on the agar following exposure to ammonia fumes (Araujo et al., 2004). Lactose-sulfite broth was suggested to have greater sensitivity than that of TSC for enumerating *C. perfringens* bacteria but was not exclusively selective for *C. perfringens*, whereas cooked meat medium was less favorable for observing *C. perfringens* growth (Xylouri et al., 1997). Therefore, in terms of differential selective media and after more than 30 years of medium innovations, TSC still appears to be best for detection and enumeration of *C. perfringens* bacteria from foods (Emswiler et al., 1977).

The many toxins produced by *C. perfringens* are listed in Table 1. Such a range of products specific to *C. perfringens* provides an ample library of antigenic determinants for subsequent immunological detection schemes. As a result, the toxinogenic typing (type A, B, C, D, or E) of *C. perfringens* is not based on the serologic specificity of *C. perfringens* enterotoxin (CPE)-related food-borne illness but on the many other exotoxins produced by the microorganism and is designated alpha, beta, epsilon, and iota (Petit et al., 1999; Brown 2000). The slide agglutination serotyping technique was developed by Stringer et al. (1980). Whole-cell preparations of isolated *C. perfringens* cultures were used as antigens against antisera raised using confirmed type strains (Stringer et al., 1980). Agglutination to specific antisera resulted in the designation of specific serotypes (Stringer et al., 1980). The mouse neutralization protection test was used initially to type *C. perfringens* strains (Sterne and Batty, 1975). Neutralization of toxin-type specific pathological effects was accomplished using appropriate antisera in laboratory mouse models (Sterne and Batty, 1975). Unfortunately, toxin-type positive results were at the expense of the animal and may be considered inhumane. Typically intestinal

fluid samples are typed by combined inoculation of known antitoxins into mice, which allows the mice to survive if protected through the neutralization of only those toxins in the intestinal fluid samples that match the known antitoxins. Although both gas-liquid chromatography and nuclear magnetic resonance spectroscopy have shown the ability to discriminate among strains of *C. perfringens* due to capsular polysaccharide characteristics, their use is of limited practical routine practice due to being laborious, expensive, and time consuming (Paine and Cherniak 1975; Sheng and Cherniak 1998).

A number of immunological tests have been evaluated for detection of *C. perfringens* type D epsilon-toxin, including a polyclonal capture enzyme-linked immunosorbent assay (PC-ELISA), a monoclonal capture ELISA (MC-ELISA), and counterimmunoelectrophoresis (CIEP) (Naik and Duncan, 1977; Uzal et al., 2003). With respect to ability to detect epsilon-toxin in the intestinal contents and body fluids of sheep and goats, the PC-ELISA was most sensitive in enabling the detection of a 0.075 50% mouse lethal dose (MLD_{50})/ml compared to the MC-ELISA (25 MLD_{50}/ml) and CIEP (50 MLD_{50}/ml) (Uzal et al., 2003). The mouse neutralization test was also used as a standard (6 MLD_{50}/ml) (Uzal et al., 2003). The sensitivity results were in marked contrast to specificity results for the PC-ELISA (31.57%), MC-ELISA (57.89%), and CIEP (84.21%) (Uzal et al., 2003). Due to the inconsistencies among the diagnostic tests for enterotoxemia from *C. perfringens* epsilon-toxin, the authors recommended that absolute confirmation should include combined clinical and pathological data as well.

Another group of researchers has developed a four-layer sandwich ELISA for the detection of type D epsilon-toxin with a sensitivity limit of 6.25 ng/ml for purified toxin preparations (El Idrissi, 1989). Double antibody sandwich techniques have been used previously (Weddell and Worthington, 1984; Naylor et al., 1987). Serological tests that have been used for epsilon-toxin detection include radial immunodiffusion and reverse passive hemagglutination (Beh and Buttery 1978). A sensitive two-site enzyme-linked immunoassay utilizing electrochemical detection has been developed for *C. perfringens* phospholipase C (alpha-toxin) at detection limits of 67.1 and 13.0 ng/ml based on use of microtiter plates and polydispersed polymeric microbeads, respectively (Cardosi et al., 2005). Western immunoblotting for CPE typically suffers from the disadvantage of isolates poorly sporulating in vitro in lab media, preventing the detection of the sporulation-associated enterotoxin (Kokai-Kun et al., 1994).

Type A food-borne CPE production is considered a relatively rare characteristic, present in approximately only 6% of tested *C. perfringens* strains (Van Damme-Jongsten et al., 1989). It has been reported that all of the food poisoning *C. perfringens* isolates carried a chromosomal *cpe* gene (Collie and McClane, 1998). There are a number of methods used for the detection of CPE in feces. The tissue culture assay using Vero cells has been determined to have relatively low sensitivity, having a limit of detection of 40 ng enterotoxin/g feces compared to an ELISA (Tech Lab, Inc.) that can detect 5 ng/g (Bartholomew et al., 1985; Berry et al., 1988). A reversed passive latex agglutination assay for CPE quantitation is also available from Oxoid. In the case of isolates (as opposed to feces), these assays depend on the in vitro sporulation of such isolates. Many strains of *C. perfringens* sporulate only reluctantly (Hsieh and Labbe, 2007).

Current innovations in epidemiological detection of food-borne CPE include a multitude of DNA-based technologies. Baez and Juneja (1995) used a filter colony hybridization assay with digoxigenin-labeled DNA specific for the *C. perfringens* type A enterotoxin gene to enumerate enterotoxigenic *C. perfringens* bacteria from raw beef. The PCR was used to amplify and incorporate digoxigenin dUTP into a 364-bp internal region of the *cpe* gene (Baez and Juneja, 1995). Following hybridization of filter membranes containing fixed DNA from *C. perfringens* cells from meat samples with anti-digoxigenin antibody conjugated to alkaline phosphatase, visualization was accomplished with a blue precipitate using the substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Baez and Juneja, 1995). The 2-day assay was sensitive enough to detect $\leq$10 CFU/g in the presence of a heterogeneous bacterial flora containing 10^6 CFU/g (Baez and Juneja, 1995). In a similar manner, 380 samples of spices and herbs were analyzed for *C. perfringens* and enterotoxigenicity with a dot-blot technique incorporating a *cpe*-gene digoxigenin-labeled DNA probe, which detected *cpe* in 4.25% of 188 confirmed isolates of *C. perfringens* (Rodriguez-Romo et al., 1998). Another similar method has been described for the enumeration and facilitated isolation of *cpe*-positive *C. perfringens* from fecal samples using a hydrophobic grid membrane filter colony hybridization method (Heikinheimo et al., 2004).

There are numerous PCR methods for detection of *C. perfringens* from foods and stool samples. DNA samples are isolated from suspected isolates of *C. perfringens* on selective agar and then amplified using specific primer pairs for a gene encoding a product such as alpha-toxin (Kalender and Ertas, 2005).

Specific PCR assays have been developed for the detection of the alpha-, beta-, and epsilon-toxin genes that offer a reliable and quicker alternative to the biological mouse assay for toxin typing of *C. perfringens* strains (Miserez et al., 1998). The presence of alpha-, beta-, beta-2-, epsilon-, iota-, and enterotoxin toxin genes in *C. perfringens* isolates from poultry across Sweden has been analyzed using PCR (Engstrom et al., 2003). Certainly with respect to specific components of foods, such as collagen, there may be inhibitory barriers to PCR use (Kim et al., 2000). Other limitations to PCR use include accurate product size determinations and nonspecific target amplifications. In addition to single gene PCR assays, multiplex PCR assays have been developed to simultaneously detect more than one gene target, such as the alpha-toxin gene present in all *C. perfringens* strains, as well as the *cpe* gene present only in food poisoning isolates of *C. perfringens* (Kanakaraj et al., 1998). Another application advantage for multiplex PCR would be the comprehensive detection of several toxin genes, such as *cpa*, *cpb*, *etx*, *iap*, *cpe*, and *cpb2*, providing very useful *C. perfringens* genotyping diagnoses (Baums et al., 2004). A quantitative real-time PCR assay, through the combination of PCR with fluorescently incorporated nucleotide base labels in the DNA amplification process for specific gene targets, enables an even more rapid detection (within a few hours) and enumeration scheme for *C. perfringens* isolates in a food or fecal sample (Wise and Siragusa, 2005). The analytical sensitivity limit of the real-time PCR assay was reported to be 100 CFU of *C. perfringens*/g in ileal samples, but 10,000 *C. perfringens* CFU/g in cecal samples as a result of unidentified PCR inhibitors (Wise and Siragusa, 2005).

Pulsed-field gel electrophoresis (PFGE) has been successfully used to type *C. perfringens* isolates (Maslanka et al., 1999). The change in the direction of the electric field and current during a preprogrammed electrophoretic migration of genomic DNA allows the separation of very large (>10-Mb) DNA fragments. Unlike standard restriction enzyme patterns for DNA agarose gel electrophoresis, restriction enzymes that cut DNA less frequently (6- to 8-bp cutters) are used in order to enhance discriminatory differences in DNA banding patterns. The inability to subtype 8% of the *C. perfringens* isolates analyzed using PFGE could be explained by the presence of strong nucleases from *C. perfringens* strains that degrade the genomic DNA preparations (Maslanka et al., 1999). PFGE has been shown to be an effective means to analyze the genetic diversity among *C. perfringens* isolates (Lin and Labbe, 2003; Nauerby et al., 2003).

With respect to extracellular DNase production limiting *C. perfringens* PFGE results, there are reports that amplified fragment length polymorphism (AFLP) analysis can provide an alternative solution to those problems (Keto-Timonen et al., 2006). In AFLP analysis the total DNA from an isolate is digested with restriction enzymes, and then adapters that allow amplification of subsets of fragments by PCR are ligated, producing a fingerprint that can be used with fluorescently labeled primers to allow computer automated data collection and database searches for comparisons (Vos et al., 1995). The AFLP procedure does not need to involve a complex mixture of complementary restriction enzymes, as a recent assay using a single restriction enzyme, Hind III, was used to provide a quick, economic method with good discrimination and reproducibility for *C. perfringens* strains of animal origin (Shinya et al., 2006). The AFLP method allowed for PCR amplification of the alpha-, beta-, epsilon-, iota-, enterotoxin, and beta-2-toxin genes of *C. perfringens* (Shinya et al., 2006). A drawback of the technique was the presence of multiple *C. perfringens* biotypes in the same animal, as well as different biotypes in the same AFLP profile that was attributed to mobile genetic elements, such as transposons, insertion sequences, plasmids, and bacteriophages (Shinya et al., 2006).

Ribotyping is a method that utilizes the highly conserved nature of ribosomal DNA sequences as a means of identifying discriminatory strain differences. Ribotyping was used to determine the genetic relationship of *C. perfringens* isolates from foodborne poisoning cases (Schalch et al., 1997). Twelve distinct ribotype patterns were discovered among 34 *C. perfringens* isolates analyzed (Schalch et al., 1997). EcoRI-digested DNA fragments were probed with digoxigenin-labeled 16S and 23S rRNA from *Escherichia coli,* resulting in ribopatterns with greater discernibility than that of the profiling of plasmid DNA (Schalch et al., 1997). Dupont Qualicon (Wilmington, DE) offers an automated RiboPrint system. Using repetitive-element PCR and EcoRI ribotyping, molecular subtyping of *C. perfringens* isolates was dramatically improved over PFGE (Siragusa et al., 2006). At the 90% correlation level, repetitive-element PCR with Dt primers demonstrated greater discrimination (0.938) than that with ribotyping (0.873) (Siragusa et al., 2006).

A further advancement of multiplex PCR amplification of DNA sequences involves the use of a microarray-based method for the characterization of six *C. perfringens* toxin genes, including the genes for iota-toxin *(iA)*, alpha-toxin *(cpa)*, enterotoxin E *(cpe)*, epsilon-toxin *(etxD)*, beta-1-toxin *(cpb1)*, and beta-2-toxin

(cpb2) (Al-Khaldi et al., 2004). Hybridization of fluorescently labeled isolate DNA to complementary single-strand DNA attached to computer chips or microscope slides enables the simultaneous screening of large numbers of *C. perfringens* genes (Al-Khaldi et al., 2004). Using this technique, 16 out of 17 *C. perfringens* strains were correctly isotyped (Al-Khaldi et al., 2004). Although recent advances in molecular typing of strain isolates have improved the identification of many *C. perfringens* specimens for epidemiological purposes, none are infallible alone. The best scheme for discriminatory trace-back of suspected food-borne contamination from *C. perfringens* strains will continue to involve a combination of multiple methods, time permitting, to allow greater confidence and confirmation of resulting strain identifications.

CONCLUDING REMARKS

The incidence of *C. perfringens* food poisoning is quite common and costly. Although somewhat fastidious in growth characteristics with the use of synthetic laboratory media, the microorganism is very prolific when found in food products. Despite the pathogen's ubiquity in the natural environment, foodborne illnesses arise from the improper handling and preparation of foods. Complete eradication of the microorganism from foods is not possible, largely due to the ability to form highly resistant spores. Control measures in place take advantage of the microorganism's limitations of growth with respect to oxygen, a_w, pH, curing salts, organic acids, and natural inhibitors. Many predictive growth models have been developed to accurately estimate *C. perfringens* survival following various types of food processing scenarios. The best strategy to control *C. perfringens* appears to be a hurdle approach combined with careful handling of foods to avoid temperature abuse. Regulatory requirements for *C. perfringens* in foods in the United States follow the USDA/FSIS compliance guidelines. Discriminatory methods for evaluation and trace-back of *C. perfringens* food-borne illness have evolved over the years from culture-based methods to serological typing to more sophisticated and rapid molecular-based technologies. Awareness, preventive measures for control, and multiple hurdles appear to provide the greatest opportunities for success.

REFERENCES

Al-Khaldi, S. F., K. M. Myers, A. Rasooly, and V. Chizhikov. 2004. Genotyping of *Clostridium perfringens* toxins using multiple oligonucleotide microarray hybridization. *Mol. Cell. Probes* **18**:359–367.

Amezquita, A., C. L. Weller, L. Wang, H. Thippareddi, and D. E. Burson. 2004. Development of an integrated model for heat transfer and dynamic growth of *Clostridium perfringens* during the cooling of cooked boneless ham. *Int. J. Food Microbiol.* **101**:123–144.

Angulo, F. L., A. C. Voetsch, D. Vugia, J. L. Hadler, M. Farley, C. Hedberg, P. Cieslak, D. Morse, D. Dwyer, and D. L. Swerdlow. 1998. Determining the burden of human illness from foodborne diseases—CDC's emerging infectious disease program Foodborne Diseases Active Surveillance Network (FoodNet). *Vet. Clin. N. Amer. Food Anim. Pr.* **14**:165–172.

Anonymous. 1995. Food poisoning—an overview. *Int. Poul. Prod.* **4**:20–21.

Aran, N. 2001. The effect of calcium and sodium lactates on growth from spores of *Bacillus cereus* and *Clostridium perfringens* in a *'sous-vide'* beef goulash under temperature abuse. *Int. J. Food Microbiol.* **63**:117–123.

Araujo, P. M. August 2007. Culture medium for the detection of *Clostridium perfringens*. European Patent EP1816209 A2.

Araujo, M., R. A. Sueiro, M. J. Gomez, and M. J. Garrido. 2004. Enumeration of *Clostridium perfringens* spores in groundwater samples: comparison of six culture media. *J. Microbiol. Methods.* **57**:175–180.

Baez, L. A., and V. K. Juneja. 1995. Nonradioactive colony hybridization assay for detection and enumeration of enterotoxigenic *Clostridium perfringens* in raw beef. *Appl. Environ. Microbiol.* **61**:807–810.

Barnes, E. M., J. E. Despaul, and M. Ingram. 1963. The behavior of a food poisoning strain of *Clostridium welchii* in beef. *J. Appl. Bacteriol.* **26**:415–427.

Bartholomew, B. A., M. F. Stringer, G. N. Watson, and R. J. Gilbert. 1985. Development and application of an enzyme linked immunosorbent assay for *Clostridium perfringens* type A enterotoxin. *J. Clin. Pathol.* **38**:222–228.

Bauer, F. T., J. A. Carpenter, and J. O. Reagan. 1981. Prevalence of *Clostridium perfringens* in pork during processing. *J. Food Prot.* **44**:279–283.

Baums, C. G., U. Schotte, G. Amtsberg, and R. Goethe. 2004. Diagnostic multiplex PCR for toxin genotyping of *Clostridium perfringens* isolates. *Vet. Microbiol.* **100**:11–16.

Bean, N. H., and P. M. Griffin. 1990. Foodborne disease outbreaks in the United States, 1973–1987: pathogens, vehicles, and trends. *J. Food Prot.* **9**:804–817.

Bean, N. H., J. S. Goulding, M. T. Daniels, and F. J. Angulo. 1997. Surveillance for foodborne disease outbreaks—United States, 1988–1992. *J. Food Prot.* **60**:1265–1286.

Beh, K. J., and S. H. Buttery. 1978. Reverse phase passive haemaglutination and single radial immunodiffusion to detect epsilon antigen of *Clostridium perfringens* type D. *Aus. Vet. J.* **54**:541–544.

Berry, P. R., J. C. Rodhouse, S. Hughes, B. A. Bartholomew, and R. J. Gilbert. 1988. Evaluation of ELISA, RPLA, and Vero cell assays for detecting *Clostridium perfringens* enterotoxin in faecal specimens. *J. Clin. Pathol.* **41**:458–461.

Blankenship, L. C., S. E. Craven, R. G. Leffler, and C. Custer. 1988. Growth of *Clostridium perfringens* in cooked chili during cooling. *Appl. Environ. Microbiol.* **53**:1104–1108.

Bradshaw, J., G. Stelma, V. Jones, J. Peeler, J. Wimsatt, J. Corwin, and R. Twedt. 1982. Thermal inactivation of *Clostridium perfringens* enterotoxin in buffer and in chicken gravy. *J. Food Sci.* **47**:914–916.

Brett, M. M. 1998. Kits for the detection of some bacterial food poisoning toxins: problems, pitfalls, and benefits. *J. Appl. Microbiol.* **84**:110S–118S.

Brown, K. L. 2000. Control of bacterial spores. *Bri. Med. Bull.* **56**:158–171.

Bryan, F. L. 1969. What the sanitarian should know about *Clostridium perfringens* foodborne illness. *J. Milk Food Technol.* **32**:381–389.

Bryan, F. L. 1988. Risks associated with vehicles of foodborne pathogens and toxins. *J. Food Prot.* **51**:498–508.

Bryan, F. L., and T. W. McKinley. 1979. Hazard analysis and control of roast beef preparation in foodservice establishments. *J. Food Prot.* **42**:4–18.

Brynestad, S., and P. E. Granum. 2002. *Clostridium perfringens* and foodborne infections. *Int. J. Food Microbiol.* **74**:195–202.

Cardosi, M., S. Birch, J. Talbot, and A. Phillips. 2005. An electrochemical immunoassay for *Clostridium perfringens* phospholipase C. *Electroanalysis* **3**:169–176.

CDC. 2000. Surveillance for foodborne-disease outbreaks—United States, 1993–1997. *MMWR Morb. Mortal. Wkly. Rep.* **49**:1–51.

Chumney, R. K., and D. M. Adams. 1980. Relationship between the increased sensitivity of heat injured *Clostridium perfringens* spores to surface-active antibiotics and to sodium chloride and sodium nitrite. *J. Appl. Bacteriol.* **49**:55–63.

Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *J. Clin. Microbiol.* **36**:30–36.

Craven, S. E. 2001. Occurrence of *Clostridium perfringens* in the broiler chicken processing plant as determined by recovery in iron milk medium. *J. Food Prot.* **64**:1956–1960.

Craven, S. E. 1980. Growth and sporulation of *Clostridium perfringens* in foods. *Food Technol.* **34**:80–87, 95.

Davidson, P. M., and V. K. Juneja. 1990. Antimicrobial agents, p. 83. *In* A. L. Branen, P. M. Davidson, and S. Salminen (ed.), *Food Additives.* Marcel Dekker, Inc., New York, NY.

Deboer, E., W. Spiegelenberg, and F. Jenssen. 1985. Microbiology of spices and herbs. *Antonie van Leeuwenhoek* **51**: 435–438.

de Jong, A. E. I., G. P. Eijhusen, E. J. F. Brouwer-Post, M. Grand, T. Johansson, T. Karkkainen, J. Marugg, P. H. in't Veld, F. H. M. Warmerdam, G. Worner, A. Zicavo, F. M. Rombouts, and R. R. Beumer. 2003. Comparison of media for enumeration of *Clostridium perfringens* from foods. *J. Microbiol. Meth.* **54**:359–366.

de Jong, J. 1989. Spoilage of an acid food product by *Clostridium perfringens, C. barati,* and *C. butyricum. Int. J. Food Microbiol.* **8**:121–132.

Duncan, C. L., and D. H. Strong. 1969. Ileal loop fluid accumulation and production of diarrhea in rabbits by cell-free products of *Clostridium perfringens. J. Bacteriol.* **100**:86–94.

Duncan, C. L., D. H. Strong, and M. Sebald. 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens. J. Bacteriol.* **110**:378–391.

El Idrissi, A. H. 1989. A 4-layer sandwich ELISA for detection of *Clostridium perfringens* epsilon toxin. FAO Corporate Document Repository. http://www.fao.org/Wairdocs/ILRI/x5489B/x5489b14.htm. Accessed 25 January 2008.

Engstrom, B. E., C. Fermer, A. Lindberg, E. Saarinen, V. Baverud, and A. Gunnarsson. 2003. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. *Vet. Microbiol.* **9**:225–235.

Emswiler, B. S., C. J. Pierson, and A. W. Kotula. 1977. Comparative study of two methods for detection of *Clostridium perfringens* in ground beef. *Appl. Environ. Microbiol.* **33**:735–737.

FDA. 2001. Limitation of growth of organisms of public health concern. 2001 Food Code. Part 3-5.

Gaze, J. E. R. Shaw, and J. Archer. 1998. *Identification and Prevention of Hazards Associated with Slow Cooling of Hams and Other Large Cooked Meats and Meat Products,* review no. 8. Campden and Chorleywood Food Research Association, Gloucestershire, United Kingdom.

Gibson, A. M., and T. A. Roberts. 1986. The effect of pH, sodium chloride, sodium nitrite, and storage temperature on the growth of *Clostridium perfringens* and fecal streptococci in laboratory media. *Int. J. Food Microbiol.* **3**:195–210.

Goepfert, J. M., and H. K. Kim. 1975. Behavior of selected foodborne pathogens in raw ground beef. *J. Milk Food Technol.* **38**:449–456.

Gough, B. J., and J. A. Alford. 1965. Effect of curing agents on the growth and survival of food-poisoning strains of *Clostridium perfringens. J. Food Sci.* **30**:1025–1028.

Hall, H. E., and R. Angelotti. 1965. *Clostridium perfringens* in meat and meat products. *Appl. Microbiol.* **13**:352–357.

Hallerbach, C. M., and N. N. Potter. 1981. Effects of nitrite and sorbate on bacterial populations in frankfurters and thuringer cervelat. *J. Food Prot.* **44**:341–346.

Harmon, S. M., D. A. Kautter, and J. T. Peeler. 1971. Improved medium for enumeration of *Clostridium perfringens. Appl. Microbiol.* **22**:688–692.

Heikinheimo, A., M. Lindstrom, and H. Korkeala. 2004. Enumeration and isolation of *cpe*-positive *Clostridium perfringens* spores from feces. *J. Clin. Microbiol.* **42**:3992–3997.

Heredia, N. L., G. A. Garcia, R. Luevanos, R. G. Labbe, and J. S. Garcia-Alvarado. 1997. Elevation of the heat resistance of vegetative cells and spores of *Clostridium perfringens* type A by sublethal heat shock. *J. Food Prot.* **60**:998–1000.

Hobbs, G., D. Cann, B. Wilson, and J. Shewan. 1965. The incidence of organisms of the genus *Clostridium* in vacuum-packed fish in the United Kingdom. *J. Appl. Bacteriol.* **28**:265–270.

Hobbs, B. C. 1979. *Clostridium perfringens* gastroenteritis, p. 131–167. *In* H. Riemann and F. L. Bryan (ed.), *Food-Borne Infections and Intoxications,* 2nd ed. Academic Press, Inc., New York, NY.

Holley R. A., A. M. Lammerding, and F. Tittiger. 1988. Microbiological safety of traditional and starter-mediated processes for the manufacture of Italian dry sausage. *Int. J. Food Microbiol.* **7**:49–62.

Hsieh, P., and R. Labbe. 2007. Influence of petone source on sporulation of *Clostridium perfringens* type A. *J. Food Protect.* **70**:1730–1734.

Huang, L. 2003a. Growth kinetics of *Clostridium perfringens* in cooked beef. *J. Food Saf.* **23**:91–105.

Huang, L. 2003b. Estimation of growth of *Clostridium perfringens* in cooked beef under fluctuating temperature conditions. *Food Microbiol.* **20**:549–559.

Huang, L. 2003c. Dynamic computer simulation of *Clostridium perfringens* growth in cooked ground beef. *Int. J. Food Microbiol.* **87**:217–227.

ICMSF. 1996. *Clostridium perfringens,* p. 112–115. *Microorganisms in Foods 5: Characteristics of Microbial Pathogens.* Blackie Academic and Professional, London, United Kingdom.

Juneja, V. K., H. Marks, and H. Thippareddi. 2008. Predictive model for growth of *Clostridium perfringens* during cooling of cooked uncured beef. *Food Microbiol.* **25**:42–55.

Juneja, V. K., and M. Friedman. 2007. Carvacrol, cinnamaldehyde, oregano oil, and thymol inhibit *Clostridium perfringens* spore germination and outgrowth in ground turkey during chilling. *J. Food Prot.* **70**: 218–222.

Juneja, V. K., M. L. Bari, Y. Inatsu, S. Kawamoto, and M. Friedman. 2007. Control of *Clostridium perfringens* by green tea leaf extracts during cooling of cooked ground beef, chicken, and pork. *J. Food Prot.* **70**:1429–1433.

Juneja, V. K., H. Thippareddi, and M. Friedman. 2006a. Control of *Clostridium perfringens* in cooked ground beef by

carvacrol, cinnamaldehyde, thymol or oregano oil during chilling. *J. Food Prot.* **69**:1546–1551.

Juneja, V. K., H. Thippareddi, M. L. Bari, Y. Inatsu, S. Kawamoto, and M. Friedman. 2006b. Chitosan protects cooked ground beef and turkey against *Clostridium perfringens* spores during chilling. *J. Food Sci.* **71**:M236–M240.

Juneja, V. K., L. Huang, and H. Thippareddi. 2006c. Predictive model for growth of *Clostridium perfringens* in cooked cured pork. *Int. J. Food Microbiol.* **110**:85–92.

Juneja, V. K. 2006. Delayed *Clostridium perfringens* growth from a spore inocula by sodium lactate in sous-vide chicken products. *Food Microbiol.* **23**:105–111.

Juneja, V. K., and H. Thippareddi. 2004a. Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *Int. J. Food Microbiol.* **93**:155–163.

Juneja, V. K., and H. Thippareddi. 2004b. Control of *Clostridium perfringens* in a model roast beef by salts of organic acids during chilling. *J. Food Saf.* **24**:95–108.

Juneja, V. K., J. S. Novak, L. Huang, and B. S. Eblen. 2003. Increased thermotolerance of *Clostridium perfringens* spores following sublethal heat shock. *Food Control* **14**:163–168.

Juneja, V. K., and H. M. Marks. 2002. Predictive model for growth of *Clostridium perfringens* during cooling of cooked cured chicken. *Food Microbiol.* **19**:313–327.

Juneja, V. K., J. S. Novak, B. S. Eblen, and B. A. McClane. 2001a. Heat resistance of *Clostridium perfringens* vegetative cells as affected by prior heat shock. *J. Food Saf.* **21**:127–139.

Juneja, V. K., J. S. Novak, H. M. Marks, and D. E. Gombas. 2001b. Growth of *Clostridium perfringens* from spore inocula in cooked cured beef: development of a predictive model. *Innov. Food Sci. Emer. Technol.* **2**:289–301.

Juneja, V. K., R. C. Whiting, H. M. Marks, and O. P. Snyder. 1999. Predictive model for growth of *Clostridium perfringens* at temperatures applicable to cooling of cooked meat. *Food Microbiol.* **16**:335–349.

Juneja, V. K., and B. S. Marmer. 1998. Thermal inactivation of *Clostridium perfringens* vegetative cells in ground beef and turkey as affected by sodium pyrophosphate. *Food Microbiol.* **15**:281–287.

Juneja, V. K., and B. S. Marmer. 1996. Growth of *Clostridium perfringens* from spore inocula in *sous-vide* turkey products. *Int. J. Food Microbiol.* **21**:115–123.

Juneja, V. K., B. S. Marmer, and J. E. Call. 1996. Influence of modified atmosphere packaging on growth of *Clostridium perfringens* in cooked turkey. *J. Food Saf.* **16**:141–150.

Juneja, V. K., and W. M. Majka. 1995. Outgrowth of *Clostridium perfringens* spores in cook-in-bag beef products. *J. Food Saf.* **15**:21–34.

Juneja, V. K., J. E. Call, B. S. Marmer, and A. J. Miller. 1994a. The effect of temperature abuse on *Clostridium perfringens* in cooked turkey stored under air and vacuum. *Food Microbiol.* **11**:187–193.

Juneja, V. K., B. S. Marmer, and A. J. Miller. 1994b. Growth and sporulation potential of *Clostridium perfringens* in aerobic and vacuum-packaged cooked beef. *J. Food Prot.* **57**:393–398.

Juneja, V. K., O. P. Snyder, and M. Cygnarowicz-Provost. 1994c. Influence of cooling rate on outgrowth of *Clostridium perfringens* spores and cooked ground beef. *J. Food Prot.* **57**:1063–1067.

Kalchayanand, N., C. P. Dunne, A. Sikes, and B. Ray. 2004. Germination induced and inactivation of *Clostridium* spores at medium-range hydrostatic pressure treatment. *Innov. Food. Sci. Emerg. Tech.* **5**:277–283.

Kalender, H., and H. B. Ertas. 2005. Isolation of *Clostridium perfringens* from chickens and detection of the alpha toxin gene by polymerase chain reaction (PCR). *Turk. J. Vet. Anim. Sci.* **29**:847–851.

Kalinowski, R. M., R. B. Tompkin, P. W. Bodnaruk, and W. P. Pruett. 2003. Impact of cooking, cooling, and subsequent refrigeration on the growth or survival of *Clostridium perfringens* in cooked meat and poultry products. *J. Food Prot.* **66**:1227–1232.

Kanakaraj, R., D. L. Harris, J. G. Songer, and B. Bosworth. 1998. Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. *Vet. Microbiol.* **63**:29–38.

Kang C. K., M. Woodburn, A. Pagenkopf, and R. Cheney. 1969. Growth, sporulation, and germination of *Clostridium perfringens* in media of controlled water activity. *Appl. Microbiol.* **18**:798–805.

Keto-Timonen, R., A. Heikinheimo, E. Eerola, and H. Korkeala. 2006. Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. *J. Clin. Microbiol.* **44**:4057–4065.

Kim, S., R. G. Labbe, and S. Ryu. 2000. Inhibitory effects of collagen on the PCR for detection of *Clostridium perfringens*. *Appl. Environ. Microbiol.* **66**:1213–1215.

Kokai-Kun, J. F., J. G. Songer, J. R. Czeczulin, F. Chen, and B. A. McClane. 1994. Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *J. Clin. Microbiol.* **32**:2533–2539.

Labbe, R. J., and V. K. Juneja. 2006. *Clostridium perfringens* gastroenteritis. *In* H. Riemann and D. O. Cliver (ed.), *Foodborne Infections and Intoxications*. Academic Press, Inc., San Diego, CA.

Labbe, R. J., and V. K. Juneja. 2002. *Clostridium perfringens*, p. 192–126. *In* D. O. Cliver and H. Riemann (ed.), *Foodborne Diseases*. Academic Press, Inc., San Diego, CA.

Labbe, R. G. 1989. *Clostridium perfringens*, p. 192–234. *In* M. P. Doyle (ed.), *Foodborne Bacterial Pathogens*. Marcel Dekker, Inc., New York, NY.

Labbe, R. G., and C. L. Duncan. 1970. Growth from spores of *Clostridium perfringens* in the presence of sodium nitrite. *Appl. Microbiol.* **19**:353–359.

Ladiges, W. C., J. F. Foster, and W. M. Ganz. 1974. Incidence and viability of *Clostridium perfringens* in ground beef. *J. Milk Food Technol.* **37**:622–626.

Li, J., S. Sayeed, and B. A. McClane. 2007. Prevalence of enterotoxigenic *Clostridium perfringens* isolates in Pittsburgh (Pennsylvania) area soils and home kitchens. *Appl. Environ. Microbiol.* **73**:7218–7224.

Lin, Y. T., and R. Labbe. 2003. Enterotoxigenicity and genetic relatedness of *Clostridium perfringens* isolates from retail food. *Appl. Environ. Microbiol.* **69**:1642–1646.

Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* **22**: 631–677.

Maslanka, S. E., J. G. Kerr, G. Williams, J. M. Barbaree, L. A. Carson, J. M. Miller, and B. Swaminathan. 1999. Molecular subtyping of *Clostridium perfringens* by pulsed-field gel electrophoresis to facilitate food-borne-disease outbreak investigations. *J. Clin. Microbiol.* **37**:2209–2214.

Maurer, A. J. 1983. Reduced sodium usage in poultry muscle foods. *Food Technol.* **37**:60–65.

Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* **5**:607–625.

Miserez, R., J. Frey, C. Buogo, S. Capaul, A. Tontis, A. Burnens, and J. Nicolet. 1998. Detection of α- and ε-toxigenic *Clostridium*

perfringens type D in sheep and goats using a DNA amplification technique (PCR). *Lett. Appl. Microbiol.* **26**:382–386.

Miwa, N., T. Masuda, A. Kwamura, K. Terai, and M. Akiyama. 2002. Survival and growth of enterotoxin-positive and enterotoxin-negative *Clostridium perfringens* in laboratory media. *Int. J. Food Microbiol.* **72**:233–238.

Naik, H., and C. Duncan. 1977. Rapid detection and quantitation of *Clostridium perfringens* enterotoxin by counterimmunoelectrophoresis. *Appl. Environ. Microbiol.* **34**:125–128.

Nauerby, B., K. Pedersen, and M. Madsen. 2003. Analysis by pulsed-field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. *Vet. Microbiol.* **94**:257–266.

Naylor, R. D., P. K. Martin, and R. T. Sharpe. 1987. Detection of *Clostridium perfringens* epsilon toxin by ELISA. *Res. Vet. Sci.* **42**:255–285.

Novak, J. S., and Y. T. C. Yuan. 2004. The fate of *Clostridium perfringens* spores exposed to ozone and/or mild heat pretreatment on beef surfaces followed by modified atmosphere packaging. *Food Microbiol.* **21**:667–673.

Oxoid Ltd. 2002. Improved detection of *Clostridium perfringens* in water samples. http://www.rapidmicrobiology.com/news/603h15.php. Accessed 25 January 2008.

Paine, C. M., and R. Cherniak. 1975. Composition of the capsular polysaccharides of *Clostridium perfringens* as a basis for their classification by chemotypes. *Can. J. Microbiol.* **21**:181–185.

Perigo, J. A., and T. A. Roberts. 1968. Inhibition of clostridia by nitrate. *J. Food. Technol.* **39**:91.

Petit, L., M. Gilbert, and M. R. Popoff. 1999. *Clostridium perfringens:* toxinotype and genotype. *Trends Microbiol.* **7**: 104–110.

Phillips, D., D. Jordan, S. Morris, I. Jenson, and J. Sumner. 2008. A national survey of the microbiological quality of retail raw meats in Australia. *J. Food Prot.* **71**:1232–1236.

Rahmati, T., and R. Labbe. 2008. Levels and toxigenicity of Bacillus cereus and Clostridium perfringens from retail seafood. *J. Food Prot.* **71**:1178–1185.

Rhodehamel, J., and S. Harmon. 1998. *Clostridium perfringens,* p. 16.01–16.06. *In* R. W. Bennett (ed.), *FDA Bacteriological Analytical Manual,* 8th ed. AOAC International, Gaithersburg, MD.

Riha, W. E., and M. Solberg. 1975. *Clostridium perfringens* inhibition by sodium nitrite as a function of pH, inoculum size, and heat. *J. Food Sci.* **40**:439–442.

Roberts, T. A., and C. M. Derrick. 1978. The effect of curing salts on the growth of *Clostridium perfringens* (welchii) in a laboratory medium. *J. Food Technol.* **13**:349–356.

Rodriguez-Romo, L. A., N. L. Heredia, R. G. Labbe, and J. S. Garcia-Alvarado. 1998. Detection of enterotoxigenic *Clostridium perfringens* in spices used in Mexico by dot blotting using a DNA probe. *J. Food Prot.* **61**:201–204.

Roy, R. J., F. F. Busta, and D. R. Thompson. 1981. Thermal inactivation of *Clostridium perfringens* after growth at several constant and linearly rising temperatures. *J. Food Sci.* **46**:1586–1591.

Sabah, J. R., H. Thippareddi, J. L. Marsden, and D. Y. C. Fung. 2003. Use of organic acids for the control of *Clostridium perfringens* in cooked vacuum-packaged restructured roast beef during an alternative cooling procedure. *J. Food Prot.* **66**:1408–1412.

Sabah, J. R., V. K. Juneja, and D. Y. C. Fung. 2004. Effect of spices and organic acids on the growth of *Clostridium perfringens* during cooling of cooked ground beef. *J. Food Prot.* **67**: 1840–1847.

Saito, M. 1990. Production of enterotoxin by *Clostridium perfringens* derived from humans, animals, foods, and the natural environment in Japan. *J. Food Prot.* **53**:115–118.

Sarker, M. R., R. P. Shivers, S. G. Sparks, V. K. Juneja, and B. A. McClane. 2000. Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. *Appl. Environ. Microbiol.* **66**:3234–3240.

Sauter, E. A., J. D. Kemp, and B. E. Langlois. 1977. Effect of nitrite and erythorbate on recovery of *Clostridium perfringens* spores in cured pork. *J. Food Sci.* **42**:1678–1679.

Schalch, B., J. Bjorkroth, H. Eisgruber, H. Korkeala, and A. Stolle. 1997. Ribotyping for strain characterization of *Clostridium perfringens* isolates from food poisoning cases and outbreaks. *Appl. Environ. Microbiol.* **63**:3992–3994.

Sheng, S., and R. Cherniak. 1998. Structure of the capsular polysaccharide of *Clostridium perfringens* Hobbs 10 determined by NMR spectroscopy. *Carbohydr. Res.* **305**:65–72.

Sheridan, J. J., R. L. Buchanan, and T. J. Montville (ed.). 1996. *HACCP: an Integrated Approach to Assuring the Microbiological Safety of Meat and Poultry.* Food & Nutrition Press, Trumbull, CT.

Shigehisa, T., T. Nakagami, and S. Taji. 1985. Influence of heating and cooling rates on spore germination and growth of *Clostridium perfringens* in media and in roast beef. *Jpn. J. Vet. Sci.* **47**:259–267.

Shinya, L. T., M. R. Baccaro, and A. M. Moreno. 2006. Use of single-enzyme amplified fragment length polymorphism for typing *Clostridium perfringens* isolated from diarrheic piglets. *Braz. J. Microbiol.* **37**:385–389.

Shoemaker, S. P., and M. D. Pierson. 1976. "Phoenix phenomenon" in the growth of *Clostridium perfringens. Appl. Environ. Microbiol.* **32**:803–807.

Siragusa, G. R., M. D. Danyluk, K. L. Hiett, M. G. Wise, and S. E. Craven. 2006. Molecular subtyping of poultry-associated type A *Clostridium perfringens* isolates by repetitive-element PCR. *J. Clin. Microbiol.* **44**:1065–1073.

Smart, J. L., T. A. Roberts, M. F. Stringer, and N. Shah. 1979. The incidence and serotypes of *Clostridium perfringens* on beef, pork and lamb carcasses. *J. Appl. Bacteriol.* **46**:377–383.

Smith-Simpson, S., and D. W. Schaffner. 2005. Development of a model to predict growth of *Clostridium perfringens* in cooked beef during cooling. *J. Food Prot.* **68**:336–341.

Solberg, M., and B. Elkind. 1970. Effect of processing and storage conditions on the microflora of *Clostridium perfringens*-inoculated frankfurters. *J. Food Sci.* **35**:126–131.

Steele, F. M., and K. H. Wright. 2001. Cooling rate effect of outgrowth of *Clostridium perfringens* in cooked, ready-to-eat turkey breast roasts. *Poult. Sci.* **80**:813–816.

Sterne, M., and I. Batty. 1975. *Pathogenic Clostridia,* p. 79–122. Butterworths, London, England.

Stringer, M. F., P. C. B. Turnbull, and R. J. Gilbert. 1980. Application of serological typing to the investigation of outbreaks of *Clostridium perfringens* food poisoning, 1970–1978. *J. Hyg.* **84**:443–456.

Strong, D. H., E. F. Foster, and C. L. Duncan. 1970. Influence of water activity on the growth of *Clostridium perfringens. Appl. Microbiol.* **19**:980–987.

Strong, D. H., K. F. Weiss, and L. W. Higgins. 1966. Survival of *Clostridium perfringens* in starch pastes. *J. Am. Diet. Assoc.* **49**:191–195.

Taormina, P. J., G. W. Bartholomew, and W. J. Dorsa. 2003. Incidence of *Clostridium perfringens* in commercially produced cured raw meat product mixtures and behavior in cooked products during chilling and refrigerated storage. *J. Food Prot.* **66**:72–81.

Thippareddi, H., V. K. Juneja, R. K. Phebus, J. L. Marsden, and C. L. Kastner. 2003. Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of ground roast beef and injected pork. *J. Food Prot.* **66**:376–381.

Todd, E. C. D. 1989. Costs of acute bacterial foodborne disease in Canada and the United States. *Int. J. Food Microbiol.* **9**:313–326.

Traci, P. A., and C. L. Duncan. 1974. Cold shock lethality and injury in *Clostridium perfringens*. *Appl. Microbiol.* **28**:815–821.

Tschirdewahn, B., S. Notermans, K. Wernars, and F. Untermann. 1991. The presence of enterotoxigenic *Clostridium perfringens* strains in feces of various animals. *Int. J. Food Microbiol.* **14**:175–178.

Tuomi, S., M. E. Matthews, and E. H. Marth. 1974. Behavior of *Clostridium perfringens* in precooked chilled ground beef gravy during cooling, holding, and reheating. *J. Milk Food Technol.* **37**:494–498.

USDA/FSIS. 2001. Performance standards for the production of certain meat and poultry products, final rule. *Fed. Regist.* **64**:732–749.

Uzal, F. A., W. R. Kelly, R. Thomas, M. Hornitzky, and F. Galea. 2003. Comparison of four techniques for the detection of *Clostridium perfringens* type D epsilon toxin in intestinal contents and other body fluids of sheep and goats. *J. Vet. Diagn. Invest.* **15**:94–99.

Van Damme-Jongsten, M., M. K. Wernars, and S. Notermans. 1989. Cloning and sequencing of the *Clostridium perfringens* enterotoxin gene. *Antonie van Leeuwenhoek* **56**:181–190.

Vareltzis, K., E. M. Buck, and R. G. Labbe. 1984. Effectiveness of a Betalains/Potassium Sorbate system versus sodium nitrite for color development and control of total aerobes, *Clostridium perfringens* and *Clostridium sporogenes* in chicken frankfurters. *J. Food Prot.* **47**:532–536.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kulper, and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acid Res.* **23**:4407–4414.

Waldroup, A. L. 1996. Contamination of raw poultry with pathogens. *World Poult. Sci. J.* **52**:7–25.

Walker, H. W. 1975. Foodborne illness from *Clostridium perfringens*. *CRC Crit. Rev. Food Sci. Nutr.* **7**:71–104.

Weddell, W., and R. W. Worthington. 1984. An enzyme labeled immunosorbent assay for measuring *Clostridium perfringens* epsilon toxin gut contents. *N. Z. Vet. J.* **33**:36–37.

Wen, Q., and B. McClane. 2004. Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Appl. Environ. Microbiol.* **70**:2685–2691.

Willardsen, R. R., F. F. Busta, and C. E. Allen. 1979. Growth of *Clostridium perfringens* in three different beef media and fluid thioglycollate medium at static and constantly rising temperatures. *J. Food Prot.* **42**:144–148.

Willardsen, R. R., F. F. Busta, C. E. Allen, and L. B. Smith. 1978. Growth and survival of *Clostridium perfringens* during constantly rising temperatures. *J. Food Sci.* **43**:470–475.

Wise, M., and G. R. Siragusa. 2005. Semi-quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. *Appl. Environ. Microbiol.* **71**:3911–3916.

Xylouri, E., C. Papadopoulou, G. Antoniadis, and E. Stoforos. 1997. Rapid identification of *Clostridium perfringens* in animal feedstuffs. *Anaerobe* **3**:191–193.

Zaika, L. L. 2003. Influence of NaCl content and cooling rate on outgrowth of *Clostridium perfringens* spores in cooked ham and beef. *J. Food Prot.* **66**:1599–1603.